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THE EFFECTS OF OESTROGENS AND OF MILD CHRONIC STARVATION ON THE WHITE RAT[1]

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Abstract

Daily injections of peanut oil for 17 or 18 days cause decrease in rate of growth of the rat, and testicular atrophy. Hence results in experiments in which peanut oil (and probably similar oils) are used as solvent vehicles for administration of material by injection may be misinterpreted. Oral administration of oestradiol (3 mgm. daily) to young mature rats for three weeks or more causes decrease in growth rate, relative decrease in size of kidneys, heart, spleen, muscle, and ovaries, little effect on the liver, marked decrease in size of testes, even to actual atrophy, and frequent enlargement of the adrenals in males, with occasional enlargement in females. The adrenals are discoloured to a maroon shade, whether enlarged or not, and evidence is advanced that they are undergoing a pathological change; any enlargement is not in the nature of hypertrophy. Oral administration of stilboestrol gives similar results. The general effects of oestrogens are more marked in male than in female animals. Loss of appetite and diminished food intake are among the general effects, but the mild chronic starvation so produced can only contribute in very minor degree to the other oestrogenic effects.

In mild chronic starvation from food restriction the liver is invariably affected, while the adrenals are never enlarged nor discoloured.

The effects of combined oestrogenic and thyroid administration seem to be neither additive nor truly antagonistic.

Introduction

Effects of Oestrogens

Published data show some lack of agreement, probably owing to such causes as method of dosage, size of dose, vehicle used for injection, variation in length of administration, variation in potency of the oestrogen used, and in age and strain of the test animal (cf. 15). The discrepancies in the published results are best revealed by considering separately the effects on growth rate, and on the different body organs. The rat is referred to, unless it is otherwise stated.

Growth rate is said to be slightly decreased by injected oestrone (8, 32), markedly decreased in young animals by oestradiol (25), and initially increased in mature females by oestradiol (23). It is decreased by stilboestrol given orally (21, 24), or by pellet implantation (22), or injected dissolved in sesame oil (20) or in corn oil (13). Stilboestrol administered orally to parent females

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depresses the growth rate of their suckling young, although growth rate is relatively less retarded in young than in mature animals (21). It is decreased to actual loss of weight by benzestrol, whether injected, given orally, or implanted in pellets (2).

The heart, kidneys, and liver are said to show a relative decrease in size in normal animals (25), and a relative or actual increase in size in gonadectomized animals (14) following treatment with oestradiol or other natural oestrogens. The spleen shows a relative decrease (25). Benzestrol is said to produce no appreciable effect on kidneys or liver (2).

The thyroid, following injections of oestradiol, is variously reported to be but little affected (25), decreased in size (7), and initially hypertrophied, then atrophied (23). Both natural oestrogens and stilboestrol are said to produce destruction of acinar walls (17). Benzestrol is said to produce no appreciable effect (2).

The ovaries are said to hypertrophy if sufficient oestrogen is injected (12) but most investigators claim that they atrophy (7, 20, 21, 22, 25, 23). Oestradiol and stilboestrol both produce atrophy of the testes (2, 25, 20, 21, 22).

(The islets of Langerhans in mice are said to enlarge following administration of oestrogen (9).)

The natural oestrogens produce enlargement of the pituitary (12, 15, 7, 23, 14, 26) except in immature rats; in these, such treatment tends to produce a decrease in size (26). Stilboestrol also produces an enlargement (20, 21, 22).

(Oestrogens are reported to cause reappearance of the *X* zone in the adrenals of mice (5), and, oppositely, to produce degenerative changes (9, 10).)

In rats, enlargement of the adrenals, usually considered to be a hypertrophy, is stated to be produced by the natural oestrogens (27, 12, 7, 25, 14, 3, 26), although some investigators report little or no effect (2, 15), and even decrease in weight (8, 26), final atrophy (23), and degeneration (11). Stilboestrol is said to cause enlargement of the rat adrenal (20, 21, 22, 13) and to produce haemorrhage into the cortex (24, 28, 16), and, on the other hand, to produce little or no effect (17); it causes enlargement of guinea-pig adrenals (1). Following administration of benzestrol there is a slight tendency to enlargement with occasional slight haemorrhage (2).

Some of the effects produced by stilboestrol have been shown to be proportional to dosage. By feeding it in drinking water in gradually increasing concentration it has been shown that growth is affected first, then the gonads (atrophy), while definitely higher dosage is needed to produce enlargement of the adrenals and pituitary (21).

In so far as it is possible to draw conclusions from majority verdicts in these discrepant reports, it seems that, for the normal rat, natural oestrogens cause decrease in growth rate, relative decrease in size of heart, kidneys, liver, and spleen, atrophy of ovaries and testes, and enlargement of the pituitary and adrenals, provided dosage be adequate. A relatively greater effect is produced on male than on female animals. Stilboestrol and benzestrol

produce effects comparable to those of natural oestrogens on growth rate, the gonads, the adrenals, and the pituitary.

It is generally agreed that many of the effects of the oestrogens are mediated through the pituitary. Adrenal enlargement (28, 27, 12, 30), depression of growth rate (24), and atrophy of the ovaries (12) are usually considered to be secondary effects of this kind, though there is evidence that body growth may be directly affected (21).

Effect of Vehicle Used to Dissolve Oestrogen for Purposes of Injection

Selye (25) injected cholesterol (presumably an inert compound) dissolved in peanut oil into young rats. His results suggest a slight decrease in weights of liver and ovaries in females, and of testes in males. These effects may well be due to the peanut oil.

Richards and Kueter (24) administered stilboestrol in oil, with resulting pronounced cloudy swelling in liver and kidneys; this effect was shown to be produced by prolonged administration of the oil alone.

Heskett and Huffman (13) injected stilboestrol in corn oil, and noted that, regardless of dosage, the animals became lethargic, their coats rough and coarse, their conjunctivae hyperaemic, and they developed a nasal discharge, diarrhoea, skin eruptions, etc., and lost appetite. The results were attributed to the stilboestrol, but not improbably were partly due to the vehicle.

Bruce and Tobin (4) record that daily injections of 0.25 to 1.0 cc. of sesame oil for several weeks produce toxic effects on normal male rats, including inhibition of growth, decreased weight of testes, and increased adrenal weight.

Combined Oestrogen and Thyroid Administration

This has some practical significance, in view of the present minor vogue of treating hyperthyroid patients with oestrogens. It is well established that administration of thyroid to rats causes decreased growth rate, and hypertrophy of kidneys, heart, and adrenals, with lessened thyroid weight. Korenschevsky and Hall (14) consider that when both thyroid and oestrogen are administered they produce co-operative activity on adrenals, liver, kidneys, and heart, and a greater loss of body weight than is produced by either alone.

Claims have been made that administration of oestrogen to the experimentally hyperthyroid animal lowers oxygen consumption and decreases the basal metabolic rate (29).

Chronic Starvation

We are only concerned here with effects in the rat following chronic starvation for a period of some weeks. Mulinos and Pomerantz (18, 19) state that such chronic inanition produces atrophy of the adrenal cortex, never marked, and chiefly affecting the cytoplasm. The adrenal medulla is virtually unaffected. Loss of weight of the thyroid, spleen, and liver is relatively greater than that of the whole body. The weight and size of the ovaries are not markedly altered; anoestrus is usual.

Present Investigation

The experiments were begun in 1941 to ascertain whether the combined effects of oestradiol and thyroid administration were or were not additive. At first the oestradiol was injected dissolved in peanut oil, but since injections of peanut oil alone were found to produce definite effects, oestradiol was thereafter given orally. It seemed to produce lessened appetite, and the food intake was found to be measurably smaller. Hence the effect of mild chronic starvation was studied for comparison. Finally the effects of oestradiol and stilboestrol were compared, special attention being given to the adrenal glands.

Experimental Part

General

Each of many separate experiments were carried out with rats of the same sex and litter, each rat isolated in a separate cage. Individual variations of growth rates and of weights of organs of litter mates kept under precisely the same conditions have necessitated grouping the animals and averaging the results of groups. The muscle weighed for comparison in the experiments has been the right anterior tibialis.

Effect of Peanut Oil

When 0.1 cc. was injected intraperitoneally daily for 17 or 18 days, either alone or as vehicle for oestradiol, and then the rats were killed and their body cavities were opened up, the organ surfaces presented a peculiar glistening appearance due to a coating of minute oil droplets; this was especially noticeable on the surfaces of the liver, kidneys, and spleen.

In a few experiments the same dosage of peanut oil was injected alone and for the same periods as when used as solvent for oestradiol. The animals so treated showed a decrease in rate of growth and in weight of testes of the same order as that produced by the oestradiol solution. For example, the initial weight of a control male rat was 54.5 gm. It gained 49 gm. in 18 days and was then killed. Its testes weighed 1.33 gm. A litter mate initially weighed 56.5 gm. After 18 days' injection of 0.1 cc. peanut oil daily, it had only gained 31.5 gm., and its testes weighed only 0.38 gm.

It therefore seemed that results from experiments with oestradiol dissolved in peanut oil might be open to misinterpretation, and that conclusions as to the effect of oestradiol could be drawn more safely if it were given orally.

Experiments with Oestradiol, Given Orally

Table I gives the average results for groups of animals of the same sex and of similar initial body weights. In each single experiment the figures for the control, or the mean figures for controls, were taken for comparison. The effect on growth was calculated as the percentage ratio of the relative increases of body weight of the experimental and control animals during the experimental period. The effects on organ weights were calculated from the figures for percentage organ weights (i) based on body weights at the beginning

TABLE I

EFFECT OF OESTRADIOL ON THE GROWTH RATE AND BODY ORGANS OF THE WHITE RAT

—	Males				Females			
	Group 1	Group 2	Group 3	Group 4	Group 1	Group 2	Group 3	Group 4
No. of treated rats	2	6	3	2	3	6	7	2
Length of treatment, days	15	21 (av.)	21 (av.)	31	15	23 (av.)	22 (av.)	31
Average initial body wt., gm.	72	60	102	106	69	64	80	105
Average gain in wt., %	55	53	37	42	80	61	38	64
Organ wt. cpd. with normal, %								
Liver	96 (112)	106 (128)	90 (116)	87 (123)	110 (119)	100 (118)	103 (124)	99 (115)
Kidneys	91 (105)	97 (118)	88 (110)	78 (111)	101 (108)	96 (113)	87 (102)	80 (93)
Heart	87 (97)	96 (117)	82 (106)	76 (108)	97 (105)	92 (107)	91 (110)	86 (100)
Spleen	82 (96)	71 (85)	69 (85)	74 (103)	84 (91)	81 (95)	85 (106)	94 (111)
Muscle	76 (88)	91 (103)	78 (96)	69 (98)	87 (97)	90 (109)	—	91 (106)
Gonads	60 (69)	23 (27)	58 (72)	25 (35)	85 (91)	65 (76)	79 (93)	74 (86)
Adrenals	98 (112)	128 (155)	130 (165)	121 (173)	107 (116)	102 (118)	106 (132)	101 (117)
Thyroid	62 (74)	119 (144)	96 (121)	71 (100)	107 (115)	103 (119)	93 (113)	80 (88)

of the experimental period, and (ii) based on the final body weights. In Table I and subsequent tables the latter values are given in parentheses.

Throughout each experiment a daily dose of 3 mgm. crystalline oestradiol was fed each experimental animal. Attempts to give it with barley meal failed, but it was fairly well taken when mixed with carrot juice (controls got the carrot juice alone).

The effect on body organs can be analysed by applying the following criteria (remembering that values in parentheses are calculated from the percentage organ weights in terms of the final body weight):—

(*A*) When percentage organ weights, referred to initial body weight (i) are greater than those of controls (and thus exceed 100), actual enlargement is indicated; (ii) are equal to those of controls (giving a value approximately 100), there is no effect.

(*B*) When percentage organ weights are less than those of controls, when referred to initial body weight, but greater, when referred to final body weight, the effect on the organ is less than that of the whole body.

(*C*) When percentage organ weights, referred to final body weight, (i) are equal to those of controls (giving a figure in parentheses approximating to 100), the effect on the organ is proportional to that on the whole body; (ii) are less than those of controls, the effect is relatively greater than that on the whole body and may indicate an actual atrophy.

Examining Table I in light of these desiderata the following conclusions seem permissible. The figures show considerable variation (the variations for individual animals in a group were still greater). There is a greater effect

on males than on females. As might be expected there seems to be a more marked effect with longer treatment, though the number of animals in individual groups is too small to stress this.

Growth rate is invariably diminished.

Liver: there is no effect in females, a slight but negligible relative loss of weight in males.

Kidneys and heart: there is a relative decrease in size, but the effect is less than that on the whole body.

Thyroid: very variable results are shown; no definite conclusion can be drawn from them.

Spleen: in females it tends to remain proportional to the whole body, in males to be more markedly affected.

Muscle: in females it is less affected than the whole body; there is a somewhat greater effect in males.

Testes: marked relative decrease in weight, and sometimes actual atrophy.

Ovaries: the effect is greater than that on the whole body, but less than on the testes.

Adrenals: usually, but not invariably, there is an actual enlargement in male animals; there is usually but little effect in females, but sometimes some degree of enlargement.

Examination of individual records shows that when the body weight of male rats at the beginning of treatment was between 50 and 60 gm., oestradiol always produced an actual atrophy of the testes so that at the end of a 21-day experiment their combined weight varied from 0.16 to 0.25 gm.; with initial body weights between 60 and 80 gm., such atrophy was only occasionally produced, although there was always an arrest of growth. With still greater initial body weights, only some arrest of growth was obtained.

The colour of the adrenals in normal rats, in our experiments, has varied from bright yellow (rare) to dull and grey yellow, sometimes with a slightly reddish tinge. In practically all the animals fed (or injected) with oestradiol the adrenals showed a definite colour change, variously recorded as grey, brown, dark brown, and most frequently as brown red or maroon. Such change of colour was noted in enlarged glands, but also in others in which there was no definite enlargement, or even slight relative loss of weight.

Appetite

In association with the striking diminution in growth rate, it was observed that the rats fed oestradiol appeared to eat less food. Hence measurement of food intake was carried out in two experiments. Each day, weighed quantities of food were given in known excess of requirements, and the amount uneaten the following morning was removed, and weighed. Correction was made for partial drying of residues by several determinations of the water content of fresh material and of mixed residues. The correction is only

approximate, so that the measurements of food consumption also are only approximate. Nevertheless they should be comparable.

In Experiment 1, during the experimental period, each of a litter of four males and five females was fed daily 20 gm. bread and 25 gm. milk.

In 13 days the two control males ate respectively 446 and 465 gm. of the mixture (average 455.5 gm.). In the same period two males receiving 3 mgm. oestradiol daily ate 361 and 360 gm. (average 360.5); their intake was thus 21% less.

The corresponding figures for two control females were 369 and 431 gm. (average 400 gm.), and for the three females fed the same dose of oestradiol 429, 382, and 343 gm. (average 385 gm.); there was only a 4% diminished intake.

In Experiment 2 a litter of nine females was similarly treated. Four controls ate in 22 days 727, 733, 741, and 824 gm. (average 756 gm.). Five animals fed oestradiol ate 577, 625, 670, 726, 730 gm. (average 666 gm.), a diminished intake of 12%.

The apparently lesser effect on appetite of females than of males agrees with the other effects recorded.

The diminished food intake, although not great, suggests that some part of the effect produced by oestradiol might be traceable to a chronic mild starvation. Hence the effects of such a mild starvation were determined.

Effects of Chronic Mild Starvation

Five experiments were carried out in each of which animals on an insufficient diet were compared with controls of the same sex and litter. The controls were fed daily 20 gm. bread and 25 gm. milk; the experimental animals 8 gm. bread and 10 gm. milk. All residual food was removed each morning: the experimental animals left little. In a sixth experiment the daily allowance of the experimental animals was lowered to 4 gm. bread and 5 cc. milk and they actually lost weight, while becoming somewhat hyperactive. The results are given in Table II.

Analysis of the figures in Table II in accordance with the principles already stated permits the following conclusions. Liver, spleen, ovaries, and thyroid show a relative lessened gain of weight greater than that of the whole body. Kidneys, heart, testes, and muscle show a relative lessened gain of weight less than, or proportional to that of the whole body. Adrenals show a lessened gain of weight relatively less than that of the whole body in males, a somewhat greater effect in females.

In chronic starvation of the duration and degree of severity tested, enlargement of adrenals never occurred while the glands always retained their normal colour.

It is to be noted that the effect on growth rate was greater than that produced by oestradiol, in our experiments, so that the effects on body organs are as great or greater than can be attributed to the potential mild starvation

TABLE II

SUMMATION OF EFFECTS OF CHRONIC STARVATION ON GROWTH AND BODY ORGANS

—	Males		Females	
	Group 1	Group 2	Group 1	Group 2
Type of starvation	Mild	Mild	Mild	Severe
No. of treated rats	3	7	5	2
Duration of expt., days	21	20-21	21	20
Average initial body wt., gm.	71	84	70	76.5
Relative increase in body wt. as cpd. with controls, %	26	19	35	−80
Organ weights cpd. with normal, %				
Liver	60 (97)	58 (88)	69 (89)	34 (97)
Kidneys	66 (107)	68 (104)	72 (95)	51 (143)
Heart	64 (106)	67 (104)	76 (100)	47 (134)
Spleen	55 (89)	58 (89)	72 (96)	31 (83)
Muscle	73 (122)	81 (124)	88 (113)	29 (79)
Gonads	82 (133)	74 (116)	61 (80)	23 (64)
Adrenals	80 (127)	84 (127)	67 (90)	41 (114)
Thyroid	52 (83)	58 (89)	59 (77)	47 (130)

of the oestradiol-fed rat. A comparison of the two sets of results indicates several definite differences. The liver is practially unaffected by oestradiol, but is very susceptible to inadequacy of food intake. Oestradiol has a marked effect on the testes, mild starvation but a slight effect. Chronic starvation never produces adrenal enlargement or colour change; oestradiol frequently produces enlargement, and usually produces colour change. Hence it seemed reasonable to conclude that the general effects produced by oestradiol are primary effects and are not appreciably affected by the loss of appetite it induces.

Effects of Stilboestrol

Pure crystalline diethylstilboestrol was used, and fed in varying doses in different experiments. Some difficulty was experienced in feeding it. It was not well taken in carrot juice, but was eaten moderately well in carrot pulp, or by alternately mixing it with carrot pulp and with bread and milk (controls being of course fed the medium alone). The results are summed up in Table III.

Comparison of Tables I and III suggests general similarity in the effects of oestradiol and stilboestrol. As with oestradiol, male animals appear to be more markedly affected than females. Markedly increased dosage does not appear to produce marked difference in results, but the number of animals in each group is too few to stress this, especially since there was not complete control of the actual amounts eaten in these oral experiments.

Stilboestrol rarely produced enlargement of the adrenals, though the same maroon discoloration was produced as with oestradiol. The apparent enlargement of the livers of female rats was probably fortuitous. The uteri of the stilboestrol animals were markedly distended (with a clear fluid); those of the oestradiol-fed animals were less affected.

TABLE III

EFFECTS OF STILBOESTROL ON GROWTH, ETC.

—	Males		Females		
	Group 1	Group 2	Group 3	Group 4	Group 5
No. of test animals	4	3	4	4	3
Daily dosage, mgm.	3	6	2	3	6
Duration of expt., days	30–35	31	22	30–35	31
Average initial body weight, gm.	111.5	109	114	101	99.5
Relative increase in body wt. as cpd. with controls, %	27	36	29	49	54
Organ wt. cpd. with normal, %					
Liver	92 (140)	93 (130)	104 (122)	120 (140)	108 (128)
Kidneys	82 (118)	77 (110)	84 (99)	96 (113)	92 (109)
Heart	84 (119)	81 (113)	98 (118)	96 (113)	90 (110)
Spleen	73 (105)	66 (92)	82 (97)	77 (90)	79 (96)
Muscle	71 (102)	72 (102)	84 (103)	89 (95)	85 (100)
Gonads	17 (24)	18 (25)	114 (137)	89 (100)	94 (111)
Adrenals	108 (154)	95 (133)	77 (91)	102 (119)	83 (98)
Thyroid	66 (94)	50 (72)	76 (90)	101 (119)	66 (76)

Effect of Oestrogens on the Adrenals

An attempt was made in some of the later experiments to ascertain the nature of the adrenal enlargement and change in colour by determination of water content. No difference in colour as produced by either of the oestrogens could be definitely asserted, though the adrenals of the oestradiol-fed animals were (perhaps imagined to be) a little greyer.

The adrenals were transferred to closed weighing bottles as rapidly as possible after dissection, weighed, and then dried at 105° C. for 24 hr. The results are shown in Table IV.

The results suggest that there is a tendency to increased water content, whether or not there is an actual enlargement of the glands.

Since it has previously been shown that, in acute starvation, enlargement of the adrenals is in the nature of a hydropic degeneration, with increased water content, and decreased number of cells in corresponding areas of the adrenal cortex (6), an attempt was made to estimate the cell numbers in corresponding areas of the adrenal cortexes of animals fed oestrogens. The technique has already been fully described (6). The cell counts were made by counting nuclei in five 1 $\times$ 10 cm. strips running inward from the periphery of the cortex in a series of photomicrographs (magnification $\times$ 200), of which three or four were made for each adrenal, selecting central sections. The figures refer to results per square cm. of the measured areas. Data are given in Table V and are for a single litter of rats.

The results in Table V indicate that the slight enlargement of the adrenals produced in a male rat by stilboestrol was accompanied by a decreased number of cells in the outer part of the cortex. Examination of the photomicrographs also indicated a definite narrowing of the glomerular zone. The relative

TABLE IV

EFFECT OF OESTROGENS ON WATER CONTENT OF ADRENALS

Sex	Controls			Stilboestrol-fed					Oestradiol-fed				
	No. of animals	Adrenal dry wt.		Daily dose	No. of animals	Adrenal size	Adrenal dry wt.		Daily dose	No. of animals	Adrenal size	Adrenal dry wt.	
		Extremes	Mean				Extremes	Mean				Extremes	Mean
		%	%	Mgm.			%	%	Mgm.			%	%
Male	5	28–32	30	3	2	Enlarged	27–28	27.5	—	—		—	—
				3	1	?		26	—			—	—
				6	2	Relatively smaller	29–30	29.5	3	2	Enlarged	25–25	25
Female	7	27–32	30	2	4	Relatively smaller	26–29	27	—			—	—
				3	3	Relatively smaller	25–28	26	—			—	—
				6	1	Relatively smaller		21	—			—	—

TABLE V

CELL COUNTS IN COMPARABLE AREAS OF ADRENAL CORTEXES

—	Rat 1, male Control	Rat 2, male Stilboestrol	Rat 3, female Control	Rat 4, female Oestradiol	Rat 5, female Oestradiol	Rat 6, female Stilboestrol	Rat 7, female Stilboestrol
Daily dose, mgm.	—	6	—	3	3	6	6
Initial body wt., gm.	109	123	86	110.5	105.5	105.5	99.5
Gain in body wt. (31 days), gm.	54	18	33	20.5	21.5	19	18
Left adrenal							
Actual wt., mgm.	13.5	19.4	24.7	30.6	27.4	19.8	23.6
Relative wt., mgm. %*	12.4	15.8	28.7	27.8	26.0	18.8	23.7
Cell count	63.2	42.6	26.1	27.3	26.0	29.9	28.0
Right adrenal							
Actual wt., mgm.	13.4	12.3	18.4	26.0	25.2	21.2	15.6
Relative wt., mgm. %*	12.3	10.0	21.4	23.5	23.9	20.1	15.7
Cell count	56.0	44.9	30.5	30.9	27.4	29.6	27.6
Both adrenals							
Actual wt., mgm.	26.9	31.7	43.1	56.6	52.6	41.0	39.2
Relative wt., mgm. %*	24.7	25.8	50.1	51.2	49.9	38.9	39.4
Cell count	59.6	43.7	28.3	29.1	26.7	29.7	27.8
Mean values				27.9		28.7	

* *To initial body weight.*

decrease in weight of the adrenals of the stilboestrol-fed female rats was not accompanied by a change in cell count. There was an occasional thinning of the glomerular zone. In female animals fed oestradiol it produced no change in the adrenal weights. There was no change in cell count, but there was a marked decrease in the width of the glomerular layer.

Effect of Combined Oral Administration of Oestradiol and Desiccated Thyroid

A few experiments were carried out to ascertain this effect. In each, animals of the same sex and litter were fed daily either (i) desiccated sheep thyroid (0.32% iodine) in daily dosage of 1 : 5000 of the (initial) body weight, or (ii) 3 mgm. oestradiol, or (iii) a combined dose of the two. One or more animals (also of this litter and sex) were used as controls.

The results were not clear cut. They were certainly not additive. Thus, since both oestradiol and thyroid feeding depress growth rate, jointly they should give a greater depression, but this was not the case. Since (at least in male animals) both tend to produce adrenal enlargement, jointly they should produce greater enlargement, but they did not do so. They both depress the relative weight of the muscle tested, and jointly should do so to a greater extent, but did not do so. They both depress the development of the testes, but jointly produced no greater effect than oestradiol alone.

Nor did the results indicate a definite antagonism between the actions of the two, except perhaps on the heart; in both sexes, instead of the enlargement produced by thyroid feeding, following the combined treatment the weight of the heart remained practically normal.

The data obtained were so indefinite that it did not seem worthwhile to carry out further experiments.

Discussion of Results

General

In determining the effects of any particular treatment on growth or on one or several of the body organs of the rat it is usually considered adequate to take a number of animals of the same sex and of approximately the same initial body weight, and to divide them into two groups, one to be treated, the other kept as controls. In recorded experiments the size of such groups varies considerably. Careful examination of our records of control animals made during the past 25 years shows very marked variations both for rate of growth and for the organ weights at any particular body weight, so that large groups of randomly selected animals are essential to permit accurate conclusions concerning qualitative differences in results, unless the observed differences are constant and large. This is still more true where attempts are made to draw conclusions as to quantitative differences.

It is generally assumed that the degree of normal variation in litter mates of the same sex is much smaller than in a random selection. Our records show that this is usually the case, and that in plotted results, lines connecting values of litter mates for a particular organ (heart, kidneys, adrenals, etc.), tend to

parallel the average curve for such an organ, so that the percentage values tend to be in good agreement. Yet there are by no means infrequent exceptions, in which the reverse of such parallelism is seen, so that even with comparisons with litter mates, the average of a number of comparisons is desirable.

In some of the experiments now recorded it is recognized that the number of animals used has not been adequate; the results in such cases have not been stressed.

Peanut Oil

The definite effects on rate of growth and on the testes following injection of peanut oil indicate that there may be danger in drawing conclusions from experiments in which this oil is used as solvent vehicle for test material. It has been pointed out that Bruce and Tobin (4) obtained very similar results with sesame oil, while some of the pathological effects reported by Heskett and Huffman (13) may well be due to injected corn oil. It seems likely that the inertness of such oils has been unwarrantedly assumed. While the amounts employed therapeutically are almost certainly without significance, those used in experiments with small animals are relatively much greater and their potential effect should not be ignored.

The Effects of Administered Oestrogens

Oestradiol, in the dosage used for the periods of time stated, produced in our experiments results almost identical with the majority verdicts set out in the introduction. Effect on liver, however, seems negligible, and actual atrophy of ovaries seems doubtful, while enlargement of the adrenals was rare in female animals. There was definitely a greater effect on males than on females. Stilboestrol produced similar effects, our results in general agreeing with those of Noble (21), who administered it in drinking water.

The Effect of Oestrogens on the Adrenals

The enlargement has usually been considered a hypertrophy. We have shown that the so-called adrenal hypertrophy produced by acute starvation is in reality a pathological process in the nature of a hydropic degeneration, and we have adduced evidence from the literature that the similar enlargement that results from thiamin deficiency is also not a hypertrophy. We have stressed that whenever experimental treatment results in marked discoloration of the adrenals, this indicates presence of a pathological process (6).

We have now shown that such discoloration follows treatment with oestrogen (oestradiol or stilboestrol), the typical colour being a maroon or grey-maroon, and differing from the dark grey or dirty grey colour typical of the adrenal of the moribund acutely starved rat. This maroon discoloration occurs whether or not the adrenal is enlarged, or is even relatively somewhat smaller than normal.

We have also adduced some evidence suggesting a tendency to increased water content, not definitely associated with enlargement. Examination of histological sections of such glands suggested presence of no marked abnor-

mality such as haemorrhage, but there was a tendency to thinning of the glomerular zone, not definitely associated with enlargement. Cell counts in comparable areas of the cortex were normal in absence of enlargement, and decreased when enlargement had occurred.

Too few determinations and examinations were made to stress these findings, but they all fit together to permit the suggestion that oestrogens produce no hypertrophy of the adrenals, but rather, in the dosage used, something in the nature of a pathological process.

Mild Chronic Starvation

Our results are in moderate agreement with those of Mulinos and Pomerantz (18, 19), which were of longer duration. The absence of enlargement of the adrenals is to be stressed. The effects produced by oestrogens include loss of appetite and diminished food intake but the mild chronic starvation resulting can only influence the oestrogen results in very minor degree.

Combined Oestrogen and Thyroid Administration

Results in the few experiments performed were not in good agreement with those of Korenschevsky and Hall (14) and indicated neither an additive effect, nor a true antagonism.

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EFFECT OF REDUCING AGENTS ON THE VIABILITY OF EQUINE ENCEPHALOMYELITIS VIRUS (EASTERN TYPE)[1]

By N. A. Labzoffsky[2]

Abstract

The present communication deals with the effect of reducing agents (cysteine hydrochloride, sodium thioglycollate, and sodium formaldehyde sulphoxylate) on the viability of equine encephalomyelitis virus (Eastern type). Cysteine hydrochloride was found to be a valuable reagent in *in vitro* studies of equine encephalomyelitis virus, because it greatly retards loss of infectivity of the virus under experimental conditions. It was observed that a virus suspension containing cysteine hydrochloride (1 : 1000) remained infective after exposure to 37° C. for 14 days, although the guinea-pig titre was reduced from 1 : 10^8 to 1 : 10^2. The same reduction in the titre of virus suspended in buffered 0.85% sodium chloride solution occurred after exposure to 37° C. for 120 hr. only, and in unbuffered 0.85% sodium chloride after 24 hr. exposure. Further, equine encephalomyelitis virus, in the presence of cysteine hydrochloride, retains its infectivity without demonstrable loss, over a pH range between 4.8 and 8.2 for 48 hr. at 37° C. The titre of equine encephalomyelitis virus is maintained at 37° C. for 48 hr. in a rather wide range of Eh, created with the aid of cysteine hydrochloride, at least in the range between -0.151 and $+0.02$ volts. On the other hand, addition of sodium formaldehyde sulphoxylate or sodium thioglycollate to a suspension of equine encephalomyelitis virus does not retard loss of infectivity of the virus. These reagents, therefore, are not suitable for the conservation of infectivity of the virus *in vitro*.

Introduction

The virus of equine encephalomyelitis, although relatively stable if kept frozen or stored in glycerol, loses infectivity quite readily, especially in high dilutions, when exposed to 37° C. or room temperature. This lability of the virus presented great difficulties in studying certain aspects of the neutralization reaction because of non-specific inactivation of the virus due to environmental factors (6). The most commonly employed diluting fluids, such as hormone broth, solutions consisting of a combination of several salts buffered to the required pH, or simple buffered physiologic solution of sodium chloride, containing 5 to 10% normal serum, although having a definite advantage over previously employed 0.85% solution of sodium chloride still provide only a moderate protection, insufficient for prolonged survival of the virus under experimental conditions. The environmental factors determining the viability of the virus *in vitro* under experimental conditions, in spite of extensive investigations, have not as yet been determined for viruses in general and no satisfactory preserving fluid has been developed. The most extensively investigated environmental factor is the pH and the stability of most of the viruses to various hydrogen ion concentrations has been definitely determined. Attempts have also been made to determine the effect of reducing agents on the viability of some of the viruses, but this question

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has not received sufficient attention from the investigators and the available information is limited and inconclusive. In this connection, Mueller (8) demonstrated that loss of infectivity of Rous chicken sarcoma virus due to oxidation upon exposure to 37° C. is deferred for more than 24 hr. and occasionally longer by addition of cysteine hydrochloride 1 : 2000. Similar results were obtained by Zinsser and Tang (13) with herpes virus. Schultz, Gebhardt, and Bullock (10) likewise found that cysteine exercises a beneficial effect on the viability of poliomyelitis virus. The results obtained by McKinnon (7) of the use of cysteine in connection with vaccinia virus are rather inconclusive. His experiments indicate that cysteine offered some slight protection when virus was stored at 2° and 5° C. and at 18° and 22° C. but at the temperature of 37° C. cysteine appeared to be detrimental to the virus. From McKinnon's presentation it appears that the experiments were conducted under aerobic conditions. Bang and Herriott (1) report of successful use of $0.1M$ cysteine for preservation of infectivity of equine encephalomyelitis virus during purification process.

The preliminary experiments conducted by the writer, using cysteine hydrochloride as a reducing agent, yielded highly encouraging results. It was found that equine encephalomyelitis virus diluted 1 : 100 would survive in the presence of 1 : 1000 cysteine hydrochloride, when exposed to 37° C. from 24 to 48 hr., without diminution in the titre, and up to two weeks with only a gradual decrease in the titre. When stored at 4° C. the above preparation has displayed a remarkable stability. It was found that after three months at refrigeration temperature there was no reduction in the titre, whereas in the control preparation (virus in buffered saline) no live virus could be detected. Even after 4½ years at 4° C. the preparation remained infective, although the titre dropped, as determined by guinea-pig inoculation, from $1 : 10^8$ to $1 : 10^4$, indicating approximately 0.1% survival. With these observations as a background, a more extensive investigation of the effect of cysteine on the viability of equine encephalomyelitis virus was planned. The possibility of developing suitable conditions for prolonged survival of the virus at 37° C. has been the subject of this investigation.

Plan of Present Investigation

With the view of determining favourable conditions for prolonged survival of equine encephalomyelitis virus under experimental conditions, the effect of reducing agents (cysteine hydrochloride, sodium thioglycollate, and sodium formaldehyde sulphoxylate) on the viability of the virus was investigated. The problem was approached from the following angles:

1. The rate of loss of infectivity of the virus particles at 37° C. suspended in buffered saline containing cysteine hydrochloride was compared with that of the virus suspended in diluents without cysteine hydrochloride.

2. An attempt was made to determine the optimal range of reducing intensity for the viability of the virus at optimum pH.

3. Investigation was made of the stability of the virus at 37° C. under conditions of optimum Eh and varying pH.

4. The effects of two other reducing agents, namely, sodium thioglycollate and sodium formaldehyde sulphoxylate were investigated for comparison.

Materials and Methods

The Virus

Equine encephalomyelitis virus, Eastern type, isolated by Schofield and Labzoffsky (9), from the blood of an infected horse at St. George, Ont., during the 1938 epizootic, was employed in this investigation. The virus has been propagated, since its primary isolation, alternately in guinea-pigs and in developing chick embryos.

The infected chick embryos, usually sixth or seventh egg passage, were harvested from the 16th to 18th hour after inoculation, when moribund or dead, then macerated, and without preliminary dilution the material was distributed in 4- to 5-cc. volumes into a number of test-tubes, plugged with cotton and stored in the freezing chamber of an electric refrigerator. A portion always was reserved for bacterial cultures. Prior to any one of the tests, one of the tubes was removed from the refrigerator, thawed at room temperature, and then centrifuged in a Swedish angle centrifuge at 4000 r.p.m. for 20 min. The supernatant fluid was collected, a portion was removed for immediate animal titration and sterility test, the rest of the fluid was frozen until the results of animal titration and sterility test were available. The end point of the virus was found to be very consistent, never being less than $1:10^8$ and occasionally $1:10^9$.

The Normal Serum

The normal serum, which gave negative neutralization reaction with the above virus, was pooled serum from a number of normal guinea-pigs of varying weights. The serum was inactivated at 58° C. for 45 min.

Diluent

The diluent was 0.85% sodium chloride either unbuffered or buffered at the required pH. The final concentration of phosphate buffer used was 0.004 *M*.

Reducing Agents

The reducing agents used were cysteine hydrochloride, supplied by Eastman Kodak Co., sodium thioglycollate, and sodium formaldehyde sulphoxylate produced by the Baltimore Biological Laboratories.

The reducing agents, for use in the experiments, were prepared as follows: 0.1 gm. of reducing agent was dissolved in 15 cc. of double distilled water, filtered through a Seitz E.K. pad previously washed with distilled water. The final volume was carefully measured and adjusted to the required pH.

For adjusting pH of a reducing agent either sodium monohydrogen phosphate or sodium dihydrogen phosphate of 0.1 *M* was used, depending on the reducing agent employed. Cysteine hydrochloride, being strongly acid, was adjusted with sodium monohydrogen phosphate, while for adjusting the pH of sodium thioglycollate and sodium formaldehyde sulphoxylate, sodium dihydrogen phosphate was used. After the pH of the reagent was adjusted the further dilutions were made in buffered saline. The volume of the reagent used for the adjustment of the pH was taken into consideration when the final concentration of the reducing agent was considered.

Test Mixtures

A given volume of the dilution of the virus in buffered saline containing 10% normal guinea-pig serum was mixed with an equal quantity of the reducing agent of desired concentration. As soon as the preparation of the mixture was completed, a sample was set aside for immediate potentiometric and animal titrations and the rest was distributed into 1 cc. ampoules with capillary necks, using a syringe and needle, then sealed and placed in an incubator at 37° C. for the required length of time. As a control the parent concentration of the virus was diluted 1 in 2 with either buffered or unbuffered physiologic salt solution, as required, so that it contained the same concentration of the virus as the test mixtures. This was also sealed in ampoules and incubated at 37° C. After the exposure of the mixtures to 37° C. for a given time, potentiometric and animal titrations of the mixtures were made. For the titration of the residual virus 10-fold dilutions of the mixtures were made in buffered physiologic saline containing 5% normal guinea-pig serum. A fresh pipette was used for making each dilution. Inoculations were made as soon as the dilutions were completed, starting with the highest dilution. The same syringe and needle were used for each series of increasing concentrations.

Test Animals

Healthy guinea-pigs weighing about 400 gm., previously used for tetanus antitoxin toxicity tests, were employed for the titration of the mixtures. All inoculations were made intracerebrally, without anaesthesia, the dose being 0.2 cc. Never fewer than two guinea-pigs were used for each dilution.

Potentiometer

All potentiometric determinations were made with Leeds and Northrop Student's Potentiometer. The glass electrode was used for measuring pH and gold for Eh measurements. Before each use, the potentiometer was checked with standard buffered solutions. Not more than five Eh determinations were made before recleaning the gold electrode with chromic–sulphuric-acid cleaning mixture.

Further details of the methods will receive mention in the description of individual experiments.

Experimental Data

Effect of Cysteine Hydrochloride on the Survival of the Virus of Equine Encephalomyelitis at 37° C.

In this experiment the comparative death rate of equine encephalomyelitis virus at 37° C. in buffered, in unbuffered physiologic solution of sodium chloride, and in buffered salt solution containing cysteine hydrochloride was investigated. Three preparations were made:

(1) Virus diluted 1 : 10^3 in unbuffered physiologic solution of sodium chloride containing normal guinea-pig serum 1 : 20.

(2) Virus diluted 1 : 10^3 in buffered physiologic solution of sodium chloride containing normal guinea-pig serum 1 : 20.

(3) Virus diluted 1 : 10^3 in buffered physiologic solution of sodium chloride, containing cysteine hydrochloride 1 : 1000 and normal guinea-pig serum 1 : 20.

A portion of each of the preparations was reserved for immediate animal titration and the remainder was distributed in a number of 1 cc. ampoules, sealed, and placed in the incubator at 37° C. At different intervals the mixtures were titrated on guinea-pigs for the residual virus. Usually a pool of not fewer than three ampoules of the same preparation was used for the titration. Three guinea-pigs were used for each dilution. The time of exposure to 37° C. and the results of animal titration are shown in Table I. No potentiometric titrations were made; the pH, however, was checked colorimetrically before and after the exposure to 37° C.

It will be seen from the table that cysteine, in the concentration used, has definitely exercised a beneficial effect on the viability of the virus. The rate of deterioration of the virus has been considerably retarded and even after 14 days of exposure to 37° C. live virus has been recovered from the initial concentration. The deterioration of the virus became demonstrable after 48 hr. at 37° C. and reached apparent equilibrium after 96 hr. of exposure. This equilibrium lasted through to the sixth day, after which the loss of infectivity of the virus particles became more accelerated. Deterioration of the virus in the buffered saline, on the other hand, was considerably faster. The titre of the virus after 48 hr. at 37° C. dropped from 1 : 10^8 to 1 : 10^6, indicating approximately 1% of survival. After 120 hr. of exposure approximately only 0.001% of the virus survived and no live virus was detected after that. In unbuffered saline, only approximately 0.0001% survived 24 hr. exposure to 37° C. and no live virus was demonstrable in the preparations exposed for 48 hr.

The results obtained in this experiment were highly encouraging and it became clearly desirable to extend this work in order to obtain more complete data on the influence of cysteine hydrochloride in maintaining the viability of the virus. For this reason in the next experiment an attempt was made to determine the effect of different reducing intensities on the viability of the virus.

TABLE I

COMPARATIVE EFFECT OF DIFFERENT DILUENTS ON THE VIABILITY OF EQUINE ENCEPHALOMYELITIS VIRUS AT 37° C.

Dilution of virus inoculated	Virus diluted 1 in 100 in:																			
	0.85% NaCl			Buffered 0.85% NaCl						Buffered 0.85% NaCl and cysteine 1 : 1000										
	Time at 37° C.																			
	0 hr.	24 hr.	48 hr.	0 hr.	48 hr.	72 hr.	96 hr.	120 hr.	6 days	0 hr.	48 hr.	72 hr.	96 hr.	120 hr.	6 days	7 days	8 days	9 days	11 days	14 days
$1:10^2$	—	3,4	S,S	—	—	—	3,3,3	3,3,S	S,S,S	—	—	—	—	—	—	3,3,3	3,3,3	3,3,3	3,3,3	3,3,S
$1:10^3$	—	S,S	S,S	—	—	3,3,4	3,3,S	3,S,S	S,S,S	—	—	—	—	—	—	3,3,3	3,4,S	3,S,S	S,S,S	S,S,S
$1:10^4$	—	S,S	—	—	3,3,3	3,3,3	3,3,4	S,S,S	—	—	3,3,3	3,3,4	3,3,3	3,3,3	3,3,4	3,3,3	S,S,S	S,S,S	S,S,S	—
$1:10^5$	—	S,S	—	—	3,3,4	3,4,S	S,S,S	S,S,S	—	—	3,3,4	3,3,3	3,3,3	3,3,4	3,3,3	3,4,S	S,S,S	—	—	—
$1:10^6$	3,3	S,S	—	3,3	3,3,3	S,S,S	S,S,S	—	—	3,3	3,3,4	3,3,3	3,4,S	3,3,3	3,3,4	—	—	—	—	—
$1:10^7$	3,3	S,S	—	3,3	S,S,S	S,S,S	—	—	—	3,3	3,3,3	3,3,4	S,S,S	S,S,S	S,S,S	—	—	—	—	—
$1:10^8$	3,3	S,S	—	3,4	S,S,S	—	—	—	—	3,3	3,3,3	3,S,S	S,S,S	—	—	—	—	—	—	—
$1:10^9$	S,S	—	—	S,S	—	—	—	—	—	S,S	—	—	—	—	—	—	—	—	—	—
% Survival	100	0.0001	0	100	1.0	0.1	0.01	0.001	0	100	100	10	1.0	1.0	1.0	1.0	0.001	0.0001	0.0001	0.0001

Note: *Number* = *day of death.*
S = *animal survived.*
— = *dilution not tested.*

Effect of Varying Reducing Intensities, Created by Different Concentrations of Cysteine Hydrochloride, at Constant pH on the Viability of Equine Encephalomyelitis Virus

In this experiment, using cysteine hydrochloride as a reducing agent, the effect of different reducing intensities on the survival of the virus at 37° C. was investigated and an attempt was made to determine an approximate Eh range for the viability of the virus. The fact that the reduction of cysteine is not truly reversible, and that the system does not fall readily into line with classical examples of reversible oxidation–reduction reactions was borne in mind. Variation of reducing intensity in different preparations in this experiment was accomplished by using different concentrations of cysteine hydrochloride, while constancy of pH in the preparations was controlled by phosphate buffer.

Three concentrations of cysteine hydrochloride, adjusted to approximately pH 6.9, were made, namely 1 : 500, 1 : 3000, and 1 : 4000. The virus was diluted 1 : 500 in buffered physiologic solution of sodium chloride of pH 6.9 and containing 10% normal guinea-pig serum. Then equal volume of the above diluted virus was mixed with equal quantity of each of the above concentrations of cysteine. As a control the parent concentration of the virus was diluted 1 in 2 with buffered physiologic saline. Thus the resulting four preparations contained final concentration of virus, $1:10^3$; normal serum, 1 : 20; cysteine: preparation No. 1, 1 : 1000; preparation No. 2, 1 : 6000; preparation No. 3, 1 : 8000; and preparation No. 4 (control) none. The final pH of the preparations was approximately 6.9.

A sample was withdrawn from each mixture for the immediate potentiometric and animal titrations and the remainder of each mixture was sealed in ampoules and placed in an incubator at 37° C. for 120 hr., after which time the potentiometric and the animal titrations were carried out. The results are recorded in Tables II and III.

The Eh of the preparations prior to the exposure to 37° C., as is shown in Table II, were −0.151, −0.0022, and + 0.02 volts. After 120 hr. at 37° C.

TABLE II

Determination of potentials of virus preparations with constant pH and varying Eh before and after exposure to 37° C. for 120 hr.

Preparation	Concentration of virus	Concentration of cysteine	Prior to exposure to 37° C.		After exposure to 37° C. for 120 hr.	
			pH	Eh in volts	pH	Eh in volts
1	1 : 1000	1 : 1000	6.9	− 0.151	6.8	+ 0.0088
2	1 : 1000	1 : 6000	6.95	− 0.0022	6.85	+ 0.0337
3	1 : 1000	1 : 8000	6.9	+ 0.02	6.9	+ 0.0349
4	1 : 1000	—	6.9	—	6.85	—

TABLE III

ANIMAL TITRATION OF VIRUS PREPARATIONS WITH CONSTANT pH AND VARYING Eh BEFORE AND AFTER EXPOSURE TO 37° C. FOR 120 HR.

Dilution of virus in inoculum	Tested immediately				Tested after 120 hr. at 37° C.			
	Concentration of virus 1 : 1000							
	Concentration of cysteine			Control without cysteine	Concentration of cysteine			Control without cysteine
	1 : 1000	1 : 6000	1 : 8000		1 : 1000	1 : 6000	1 : 8000	
1 : 10^3	—	—	—	—	3,3,4	3,3,3	3,3,4	S,S,4
1 : 10^4	—	—	—	—	3,3,3	3,3,4	3,3,3	S,S,S
1 : 10^5	—	—	—	—	3,3,4	3,3,3	3,3,3	S,S,S
1 : 10^6	3,3	3,3	3,4	3,4	4,S,S	S,S,S	S,S,S	S,S,S
1 : 10^7	3,3	3,4	3,3	3,3	S,S,S	S,S,S	S,S,S	S,S,S
1 : 10^8	3,4	3,3	3,3	3,3	S,S,S	S,S,S	S,S,S	—
1 : 10^9	S,S	S,S	S,S	S,S	—	—	—	—

Note: Number = day of death.
S = animal survived.
— = dilution not tested.

the Eh of the mixtures has changed to + 0.0088, + 0.0337, and +0.0349 volts, respectively, while the pH was apparently unaffected or only slightly so.

The titre of the virus, as determined by guinea-pig inoculation, Table III, was the same in all three preparations containing cysteine hydrochloride, indicating that approximately the same percentage, namely 1%, of the virus survived in each of the preparations. In buffered saline control mixture no live virus was detected.

The results thus would indicate that the viability of the virus of encephalomyelitis is maintained in a rather wide range of Eh, at least in the range between −0.151 and + 0.02 volts, providing the pH of the preserving fluid is kept around 7. The results also provide further proof of the beneficial influence of cysteine in maintaining the viability of the virus. This becomes especially impressive when high initial dilution of the virus is taken into consideration and the fact that no live virus was detected in the control preparation.

In the next experiment the effect of different pH values on the survival of the virus in the presence of cysteine hydrochloride is investigated.

Effect of Cysteine Hydrochloride on the Stability of Infectivity of Equine Encephalomyelitis Virus at Various Hydrogen Ion Concentrations

Effect of pH on the viability of different viruses under experimental conditions has been extensively investigated during recent years. The results of the detailed study of this question in case of plant viruses (11, 12) and some of the animal viruses (2, 3) indicate that for each individual virus studied there is a definite pH zone in which the stability of a given virus is most

pronounced. On either side of this zone there is a relatively rapid drop in stability. The virus of equine encephalomyelitis (Eastern type), however, as reported by Finkelstein and his co-workers (4, 5) appears to behave in a different manner. Their results show that at 0° C. to 5° C. the greatest stability range of infectivity of the above virus lay between pH 7.5 and pH 8.5. A second range of relative stability was found to be between pH 3.5 and pH 5.0. Between these two extreme ranges there is a region of rapid inactivation between pH 5.2 and pH 5.8, the extreme being at pH 5.5. From a practical standpoint it was felt desirable to determine whether or not this stability of equine encephalomyelitis virus in relation to pH is in any way influenced by the addition of cysteine hydrochloride. The pH range chosen for this study covers three points relative to the stability of the virus, namely, pH 4.8 (point of relative stability), pH 5.6 (point in a region of rapid inactivation), and pH 8.2 (point in the region of maximum stability).

Three buffer–virus mixtures with above pH values were prepared. Each mixture contained final concentration of virus 1 : 10^4 and normal guinea-pig serum 1 : 20. In order to have the initial Eh approximately the same in each of the three mixtures, the concentration of cysteine hydrochloride had to be varied, thus Mixture I of pH 4.8 had concentration of cysteine 1 : 600, giving Eh of the mixture −0.1690 volts. Mixture II of pH 5.6 contained cysteine 1 : 800 and had Eh −0.1685 volts. Mixture III of pH 8.2 contained cysteine 1 : 1200, and had Eh −0.1705 volts. As a control three buffer–virus mixtures with corresponding pH values were prepared.

The results of the potentiometric and animal titration of the mixtures before and after the exposure to 37° C. for 48 hr. are given in Tables IV and V.

TABLE IV

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS WITH CONSTANT Eh AND VARYING pH VALUES BEFORE AND AFTER EXPOSURE TO 37° C. FOR 48 HR.

Virus preparation	Concentration of virus	Concentration of cysteine	Prior to exposure to 37° C.		After 48 hr. at 37° C.	
			pH	Eh in volts	pH	Eh in volts
I	1 : 10,000	1 : 600	4.8	−0.1690	4.85	−0.0675
II	1 : 10,000	1 : 800	5.6	−0.1685	5.75	−0.045
III	1 : 10,000	1 : 1200	8.2	−0.1705	8.25	−0.0811
IV	1 : 10,000	—	4.8	—	4.75	—
V	1 : 10,000	—	5.6	—	5.8	—
VI	1 : 10,000	—	8.2	—	8.3	—

It will be seen from Table IV that the pH of the mixtures was practically unaffected by the exposure to 37° C., the slight difference recorded might have been due to a technical inaccuracy during the determination. Eh of the mixtures, on the other hand, has changed considerably, especially in Mixture II. The final Eh of the mixtures, however, remained on the negative side.

TABLE V

COMPARATIVE TITRE OF THE VIRUS IN THE PREPARATIONS WITH CONSTANT Eh AND VARYING pH VALUES AFTER EXPOSURE TO 37° C. FOR 48 HR., AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Initial concentration of virus 1 : 10,000					
		Preparations containing cysteine			Control virus preparation in buffered saline		
		I 1 : 600 pH 4.85	II 1 : 800 pH 5.75	III 1 : 1200 pH 8.25	IV pH 4.75	V pH 5.8	VI pH 8.3
1 : 1	1 : 10^4	3,3,3	3,3,4	3,3,4	3,4,S	5,S,S	3,3,4
1 : 10	1 : 10^5	3,3,3	3,3,3	3,3,4	S,S,S	S,S,S	3,4,4
1 : 10^2	1 : 10^6	3,3,4	3,3,4	3,3,3	S,S,S	S,S,S	S,S,S
1 : 10^3	1 : 10^7	3,4,4	3,3,4	3,3,4	S,S,S	S,S,S	S,S,S
1 : 10^4	1 : 10^8	3,3,4	3,4,4	3,3,4	—	—	—
1 : 10^5	1 : 10^9	S,S,S	S,S,S	S,S,S	—	—	—
Initial titre of the preparations prior to exposure to 37° C. as determined by guinea-pig inoculation		1 : 10^8	1 : 10^8	1 : 10^8	1 : 10^8	1 : 10^8	1 : 10^8

Note: *Number = day of death.*
S = animal survived.
— = dilution not tested.

This change of Eh apparently had no detrimental effect on the viability of the virus as was shown by animal titration of the residual virus. The guinea-pig titre of all three mixtures after exposure to 37° C. for 48 hr. was found to be the same, 1 : 10^8, indicating that approximately the same percentage of the virus has survived in each case. The titre of the control mixtures, however, has dropped considerably in comparison to the test preparations. This is especially pronounced in Mixture V with initial pH 5.6 where only one animal out of three inoculated with the highest concentration of the virus succumbed. In Control Mixture IV of pH 4.75 approximately 0.01% of the virus survived and in Mixture VI of pH 8.2 the approximate survival of the virus was 0.1%, that is 10 times as much as in Mixture IV. The results of the control mixtures are in close agreement with those obtained by Finkelstein and his co-workers (4, 5). On the whole the data obtained would indicate that addition of cysteine hydrochloride to the virus suspension minimized the effect on the virus of pH in the range studied. The virus appears to survive equally well for at least 48 hr. at 37° C. in pH range between 4.8 and 8.2. This fact may prove to be of significance when effect of the pH on the neutralization reaction is determined and also in connection with purification of the virus.

In the succeeding section the effect of other reducing agents on the virus is reported.

Effect of Sodium Thioglycollate and Sodium Formaldehyde Sulphoxylate on the Viability of Equine Encephalomyelitis Virus

In this series of experiments the effect of sodium thioglycollate and sodium formaldehyde sulphoxylate on the virus in question is investigated. Both these substances are now extensively employed in connection with cultivation of anaerobic bacteria and it was thought desirable to determine whether or not these reagents could be employed in the preservation of the virus. With this in view several different concentrations of these two substances were tested. Two experiments were conducted employing sodium formaldehyde sulphoxylate and one with sodium thioglycollate.

The final concentration of virus in all three experiments was $1:10^3$. The final concentration of sodium formaldehyde sulphoxylate in the first experiment was 1 : 800 and 1 : 3000; in the second 1 : 2500, 1 : 5000, and 1 : 10,000. The concentrations of sodium thioglycollate tested were 1 : 400 and 1 : 800. As a control, a suspension of virus of the same concentration in buffered saline was used. In the first experiment with sodium formaldehyde sulphoxylate the mixtures were exposed to 37° C. for 96 hr.; in the second and in the experiment with sodium thioglycollate the exposure was 72 hr. The results of potentiometric and animal titrations both before and after the exposure to 37° C. are reported in Tables VI, VII, VIII, IX, X, and XI.

In the first experiment with sodium formaldehyde sulphoxylate, Tables VI and VII, no live virus was detected, after the exposure to 37° C. for 96 hr., in the preparation containing the above reagent 1 : 800 nor in the control preparation. In the preparation containing the reagent 1 : 3000 only one guinea-pig out of two came down with encephalomyelitis in the dilution $1:10^4$. The results thus may indicate that some slight protection was offered by the above substance in the concentration of 1 : 3000. In the second experiment the titre of the virus, as determined by guinea-pig inoculation, in all three test mixtures containing different concentrations of sodium formaldehyde sulphoxylate, namely, 1 : 2500, 1 : 5000, and 1 : 10,000, was the same and somewhat higher than in the control, indicating some slight protection. The protective effect, however, was not as striking as that with cysteine hydrochloride. The results of the second experiment might be somewhat misleading and more striking difference between the titres of the test and control preparations might have been obtained if the time of the exposure to 37° C. was increased beyond 72 hr.

The results of the experiment with sodium thioglycollate appear to indicate that this reagent, besides having a low reducing intensity, is detrimental to the virus in a concentration of 1 : 400. The survival of the virus in the preparation containing the above reagent 1 : 800, Tables X and XI, taking into consideration the control preparation, may be attributed to the presence of buffer rather than to the effect of sodium thioglycollate. Neither of these reagents, therefore, compare favourably with cysteine hydrochloride.

TABLE VI

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS CONTAINING DIFFERENT CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 96 HR.

Preparation	Sodium formaldehyde sulphoxylate	Prior to exposure to 37° C.		After 96 hr. at 37° C.	
		pH	Eh in volts	pH	Eh in volts
I	1 : 800	7.5	− 0.1575	7.85	− 0.0742
II	1 : 3000	7.5	+ 0.0075	7.79	+ 0.0252
III	None	7.5	—	7.2	—

TABLE VII

TITRE OF THE VIRUS IN PREPARATIONS CONTAINING DIFFERENT CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 96 HR. AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Tested immediately			Tested after exposure to 37° C. for 96 hr.		
		Initial concentration of virus 1 : 1000 in:					
		Sodium formaldehyde sulphoxylate		Buffered saline	Sodium formaldehyde sulphoxylate		Buffered saline
		1 : 800	1 : 3000	—	1 : 800	1 : 3000	—
1 : 1	1 : 10^3	—	—	—	S,S,S	3,3,3	S,S,S
1 : 10	1 : 10^4	—	—	—	S,S,S	4,S,S	S,S,S
1 : 10^2	1 : 10^5	—	—	—	S,S,S	S,S,S	S,S,S
1 : 10^3	1 : 10^6	3,3	3,4	3,3	S,S,S	S,S,S	S,S,S
1 : 10^4	1 : 10^7	3,4	3,3	3,3	S,S,S	S,S,S	S,S,S
1 : 10^5	1 : 10^8	3,3	3,3	3,3	—	—	—
1 : 10^6	1 : 10^9	S,S	S,S	S,S	—	—	—

Note: *Number = day of death.*
S = animal survived.
— = dilution not tested.

TABLE VIII

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR.

Preparation	Concentration of virus	Sodium formaldehyde sulphoxylate	Prior to exposure to 37° C.		After exposure to 37° C. for 72 hr.	
			pH	Eh in volts	pH	Eh in volts
I	1 : 1000	1 : 2500	7.5	+ 0.004	7.3	+ 0.0021
II	1 : 1000	1 : 5000	7.5	+ 0.02	7.7	+ 0.033
III	1 : 1000	1 : 10,000	7.5	+ 0.0343	7.2	+ 0.041
IV	1 : 1000	—	7.5	—	7.4	—

TABLE IX

TITRE OF THE VIRUS IN PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM FORMALDEHYDE SULPHOXYLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR. AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Initial concentration of virus 1 : 1000 in:							
		Sodium formaldehyde sulphoxylate						Buffered saline	
		1 : 2500		1 : 5000		1 : 10,000			
		Tested							
		Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.
1 : 1	1 : 10^3	—	3,3	—	3,3	—	3,3	—	3,3
1 : 10	1 : 10^4	—	3,3	—	3,3	—	3,3	—	3,3
1 : 10^2	1 : 10^5	—	3,4	—	3,3	—	3,4	—	3,4
1 : 10^3	1 : 10^6	3,4	3,3	3,3	3,3	3,3	3,3	3,3	S,S
1 : 10^4	1 : 10^7	3,3	S,S	3,4	3,3	3,3	S,S	3,4	S,S
1 : 10^5	1 : 10^8	3,4	S,S	3,3	3,4	3,4	S,S	3,3	S,S
1 : 10^6	1 : 10^9	S,S	—	S,S	—	S,S	—	S,S	—

Note: Number = day of death.
S = animal survived.
— = dilution not tested.

TABLE X

DETERMINATION OF POTENTIALS OF VIRUS PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM THIOGLYCOLLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR.

Preparation	Concentration of virus	Sodium thioglycollate	Prior to exposure to 37° C.		After exposure to 37° C. for 72 hr.	
			pH	Eh in volts	pH	Eh in volts
I	1 : 1000	1 : 400	7.5	+ 0.032	7.65	+ 0.0435
II	1 : 1000	1 : 800	7.5	+ 0.0345	7.75	+ 0.0485
III	1 : 1000	—	7.5	—	7.45	—

Discussion

Aside from providing evidence for the beneficial influence of cysteine hydrochloride on the viability of the virus of equine encephalomyelitis (Eastern type) the results reported raise problems concerning the electrode potential of the virus suspensions.

The beneficial effect of the addition of cysteine to the virus suspensions has been demonstrated quite conclusively, as the rate of loss of infectivity of the virus in the surroundings of high reducing intensity was considerably retarded. It also has been shown that the virus will survive in a rather wide

TABLE XI

TITRE OF THE VIRUS IN PREPARATIONS CONTAINING VARYING CONCENTRATIONS OF SODIUM THIOGLYCOLLATE, BEFORE AND AFTER EXPOSURE TO 37° C. FOR 72 HR. AS DETERMINED BY INTRACEREBRAL INOCULATION INTO GUINEA-PIGS

Dilution of preparation that was inoculated	Dilution of virus in inoculum	Initial concentration of virus 1 : 1000 in:					
		Sodium thioglycollate				Buffered saline	
		1:400		1:800			
		Tested					
		Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.	Immediately	72 hr. at 37° C.
1 : 1	1 : 10^3	—	S,S	—	3,3	—	3,3
1 : 10	1 : 10^4	—	S,S	—	3,4	—	3,3
1 : 10^2	1 : 10^5	—	S,S	—	S,S	—	S,S
1 : 10^3	1 : 10^6	3,3	S,S	3,3	S,S	3,3	S,S
1 : 10^4	1 : 10^7	3,4	S,S	3,3	S,S	3,4	S,S
1 : 10^5	1 : 10^8	3,4	—	3,4	—	3,3	—
1 : 10^6	1 : 10^9	S,S	—	S,S	—	S,S	—

Note: Number = day of death.
S = animal survived.
— = dilution not tested.

range of electrode potential, at least between -0.151 and $+0.2$ volts. The findings regarding the viability of the virus at different H-ion concentrations, when Eh was kept at a low level, is of some practical interest. Although the pH range studied was rather limited, yet it serves to indicate the possibility of the virus surviving at even greater pH range to be of practical value in connection with purification and concentration of the virus. The presence of cysteine in the virus suspension appears to minimize the effect of pH to a great extent, since it was found that the virus survived equally well in the pH range between 4.8 and 8.2. The electrode potential of the virus suspensions was found to be always higher after prolonged exposure to 37° C. and no evidence of static equilibrium of the potential was observed in any of the experiments. Whether this rise of the potential was actually associated with the deterioration of the virus remains to be determined. Therefore, from the present data it is not at all clear what relation the potential of the virus suspensions measured bears to conditions within the living virus particle. This is obviously one of the fundamental problems.

The two other reducing agents, sodium thioglycollate and sodium formaldehyde sulphoxylate, employed in this study did not prove to be adequate for the purpose of preservation of the virus. Sodium formaldehyde sulphoxylate gives some slight protection, but hardly sufficient to be of practical value. Sodium thioglycollate, on the other hand, offers no protection, in fact, at least in the concentration of 1 : 400, it appears to be detrimental to the virus.

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